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Metabolism-Related Assays and Their Application to Agrochemical Research: Reactivity of Pesticides with Glutathione and Glutathione Transferases†

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Abstract: An HPLC-based assay system has been developed to measure the reactivity of agrochemicals with glutathione (GSH) with and without catalysis by glutathione transferases (GSTs). Metabolism-related parameters based on second-order related rate constants from non-enzymatic GSH and enzymatic GSH + GST assays have been derived for use in structure–activity and structure–reactivity relationship studies of exploratory agrochemicals. The versatility and sensitivity of the assay system has been established using a diverse range of agrochemicals and model compounds, e.g. 4-nitrobenzyl chloride, 1-chloro-2,4-dinitrobenzene, atrazine, acetochlor, fluorodifen, fluazifop-butyl, tridiphane, fluazinam, chlorothalonil and diazinon. For the enzymatic GSH + GST assay, second-order related rate constants, ratioed to the assay standard, 4-nitrobenzyl chloride to provide a parameter independent of assay conditions, spanned five orders of magnitude, fluazinam being the most reactive and atrazine the least. Within chemical classes significant variations in reactivity were observed, alachlor being *c.*15-fold more reactive than pretilachlor. Applications of this assay system based on comparative measures of reactivity across and within chemical classes are discussed. © 1998 Society of Chemical Industry

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Key words: glutathione; glutathione transferase; structure–reactivity relationships; herbicides; insectides; fungicides; metabolism-related parameters; HPLC assay

1 INTRODUCTION

Enzymatic catalysis of glutathione conjugation was first reported in 1961 and the purification and identification of glutathione transferases established during 1967–1974.[1,2] Since 1965 over 1500 papers attest to the potential for glutathione-dependent reactions to contribute to the biological action or fate of agrochemicals (for reviews see References 3–7). Glutathione (GSH; γ-glutamyl-cysteinyl-glycine), usually cited as the most abundant non-protein thiol in mammalian, plant, insect, fungal and bacterial cells, plays an important role in many cellular processes, including defence mechanisms against xenobiotics and environmental stress.[3,8,9] Intracellularly, glutathione exists predominantly in the reduced form (GSH) with the potential to react with agrochemicals to form hydrophilic thioether conjugates via the thiolate anion. Although direct GSH conjugation reactions can occur in biological systems, they are more generally catalysed by ubiquitous glutathione transferases (GSTs; EC 2.5.1.18). GSTs exist in multiple forms, most isoforms sharing a high specificity for glutathione or, as found in some leguminous plants, homoglutathione (hGSH) where glycine is replaced by β-alanine. In contrast, the specificity of GSTs for xenobiotics is relatively low, GSTs having a wide and overlapping ability to bind compounds of diverse structures

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and physical chemical properties.[10–13] For conjugation to occur, the xenobiotic requires an electrophilic site,[11] an intrinsic feature of many commercial pesticides,[3] e.g. the herbicides atrazine, acetochlor, metolachlor, fomesafen, fluorodifen, fluazifop-butyl and tridiphane, the fungicides chlorothalonil and fluazinam, and the insecticide diazinon. Alternatively, an electrophilic site can be introduced by oxidative metabolism, e.g. the sulfoxide of the *S*-ethyl dipropylthiocarbamate herbicide, EPTC.

Agrochemical research programmes seeking to understand degradation, selectivity and resistance mechanisms of pesticides could obviously benefit from measurements of intrinsic glutathione-dependent reactivity to explore structure–activity and structure–reactivity relationships. To this end, a versatile HPLC-based assay system has been developed to measure the reactivity of agrochemicals with glutathione, with and without catalysis by glutathione transferases.

2 EXPERIMENTAL METHODS

2.1 Materials

All agrochemicals are listed in the Pesticide Manual[14] and were used as supplied from the Zeneca Agrochemicals company collection. 4-Nitrobenzyl chloride, 1-chloro-3,4-dinitrobenzene, 1-chloro-2,4-dinitrobenzene and *N*,*N*-dipropyl-2-chloroacetamide were used as received from commercial suppliers. Organic solvents were HPLC grade and water was purified *via* a Barnstead NANOpure II system. Glutathione (G4251) and glutathione transferase from rat liver (G8386, *c*.75% protein) were obtained from Sigma.

2.2 Hydrolysis, non-enzymatic GSH and enzymatic GSH + GST assays

Compounds were dissolved in acetonitrile to give 1 g litre^{-1} stock solutions. Glutathione was dissolved in nitrogen-saturated water containing EDTA (1 mM) to give 0·1 M stock solutions which were stored for up to 10 h at 4°C. Glutathione transferase was dissolved in water or buffer solution to give 1 g litre^{-1} stock solutions which were stored at 4°C for up to one month. Buffer solutions (50 mM) at pH 7·0, 9·0 and 10·0 containing EDTA (1 mM) were prepared by dissolving one Russell Buffer capsule in water (100 ml), the pH being adjusted as required with sodium hydroxide or hydrochloric acid.

The assay system was developed to give simultaneous measurements of hydrolysis, non-enzymatic GSH and enzymatic GSH + GST reactivity, utilising the ability of modern programmable HPLC autosamplers to thermostat and randomly access sample vials. A complete assay at a given temperature (4–40°C) required four HPLC vials per compound:

(1) Mobile phase control:
mobile phase (2 ml) + compound (2–40 µl).
(2) Hydrolysis control:
buffer (2 ml) + compound (2–40 µl)
(3) Non-enzymatic GSH reactivity:
buffer (1·9 ml) + GSH (100 µl) + compound (2–40 µl)
(4) Enzymatic GSH + GST reactivity:
buffer (1·7 ml) + GSH (100 µl) + GST (200 µl) + compound (2–40 µl)

Reactions were started by addition of compound and followed by determining compound loss versus time by reverse-phase HPLC. Normally, the enzyme-catalysed reaction (4) was started first and a series of initial and repeat injections ($\leq$100 µl) made directly from the vial into the HPLC system at time intervals of 3, 15 and 30 min. The same procedure was then followed sequentially for the non-enzymatic GSH reaction (3), hydrolysis (2) and mobile phase (1) controls. If the level of compound loss in reaction (4) was observed to be $<$50% at 30 min, further samples for analysis were taken from vials (4), (3), (2) and (1) at 1, 2, 4, 8 and 16 h. For more highly reactive compounds, repeat assays were usually carried out using a lower amount of GST (minimum 0·005 mg ml^{-1} reaction solution) and shorter sampling time intervals. When the level of compound loss at 16 h was $<$50%, assays were repeated using higher amounts of GST (maximum 0·94 mg ml^{-1} reaction solution).

The HPLC system consisted of a Waters model 510 isocratic pump, 715 or 717 Autosampler with thermostatic control and 490 four-wavelength detector fitted with a BBC SE460 four-pen chart recorder and Spectra-Physics Chrom-Jet integrator. Hichrom columns (Sperisorb, S5ODS2, 4·6 × 250 mm) were used with acetonitrile + acidified water (10 g litre^{-1} phosphoric acid) mobile phases (90 + 10 to 30 + 70 by volume) at flow rates of 1 to 2 ml min^{-1}. Peak detection was at three to four wavelengths in the 210 to 300 nm range and peak heights and/or areas at an optimal wavelength were used to quantify amount of compound for a given sample time relative to the initial time sample. The mobile-phase control was used to check for variability in the analytical performance of the HPLC system. Spectrophotometric assays (2 ml) were carried out in cuvettes (quartz 1 cm pathlength) using a Perkin Elmer Lambda 17 spectrophotometer fitted with a Haake D8 circulating water bath.

2.3 Data analysis

Initial, and five to eight further measurements of change in peak height, peak area or absorbance over two to

three compound half-lives, or up to 16 h were used to determine first-order rate constants (s^{-1}) and half-lives ($t_{1/2}$) from exponential fits.[15]

Second-order rate constants for non-enzymatic reactivity of compound with GSH ($M^{-1}\ s^{-1}$) and second-order related rate constants for enzymatic reactivity of compound with GSH + GST ($M^{-1}\ s^{-1}$ (mg GST $ml^{-1})^{-1}$) were obtained by dividing corrected first-order rate constant data (GSH-hydrolysis) or (GST-GSH-hydrolysis) by the initial concentrations of GSH (M) and GST (mg ml^{-1} assay solution) as appropriate. For the enzymatic GSH + GST assay, second-order rate constant data ($M^{-1}\ s^{-1}$ (mg GST $ml^{-1})^{-1}$) were normalised by dividing the value by that obtained under the same test conditions in the same assay session for the standard (4-nitrobenzyl chloride, PNBC). This procedure minimised potential rate-constant variations due to session-to-session experimental differences in reactant concentrations, pH, temperature and HPLC system performance.

Specific activities (mole $min^{-1}\ mg^{-1}$) for the enzymatic GSH + GST assay were obtained by multiplying corrected first-order rate constants (min^{-1}) by the initial amount of compound (mole) and dividing by the amount (mg) of GST protein used.

3 RESULTS

3.1 Non-enzymatic GSH and enzymatic GSH + GST reaction kinetics

Comparative rate constants were determined under as near physiologically relevant kinetic conditions as possible. Two approaches to deriving reactivity parameters for use in structure–activity and structure–reactivity studies were considered, based on second-order rate constant or specific activity data.

3.1.1 Second-order rate constants

The non-enzymatic GSH and enzymatic GSH + GST assays were normally carried out under pseudo first-order kinetic conditions of low compound concentration (*c.* $\leqslant 100\ \mu M$) and high GSH concentration (*c.*5 mM), relevant to mammals, plants and insects.[3,8–10] Under these conditions, good exponential fits of assay data were obtained to give pseudo first-order rate constants, independent of compound concentration, but dependent upon both GSH and GST concentration, enabling second-order related rate constants to be obtained. Representative non-enzymatic GSH and enzymatic GSH + GST reactivity data for the assay standard 4-nitrobenzyl chloride (PNBC) and levels of experimental error are given in Table 1. No loss of PNBC was observed in the hydrolysis control assays.

The mean non-enzymatic GSH and enzymatic GSH + GST assay second-order related rate constants at pH 7·0 and 22°C for PNBC were 0·005 $M^{-1}\ s^{-1}$ and 25 $M^{-1}\ s^{-1}$ (mg GST $ml^{-1})^{-1}$, respectively. The ratio of enzymatic GSH + GST to non-enzymatic GSH rate constants can be used as a measure of GST catalytic efficiency, PNBC having a value of 5000 under the assay conditions used.

3.1.2 Specific activities

Enzymatic reaction rates are more usually reported in terms of specific activities in units of mole $min^{-1}\ mg^{-1}$ protein. At enzyme-saturating substrate concentrations, reaction rates expressed in these units will be at a maximum value (V_{max}) and independent of substrate concentrations. Under normal enzymatic GSH + GST assay conditions GSH concentrations (5 mM) are expected to be saturating. However, compound concentrations ($\leqslant 100\ \mu M$) were assumed to be significantly below their K_m values, leading to good exponential fits of experimental data, reaction rates in these units (mole $min^{-1}\ mg^{-1}$) being dependent upon compound concentration.

This assumption was supported by data from spectrophotometric assays using the standard PNBC as substrate with GSH (4·8 mM) and GST (0·005 mg ml^{-1}) with various concentrations of PNBC (0·05, 0·09, 0·19, 0·48 and 0·95 mM) at pH 7·0, 25°C. An excellent fit to Michaelis–Menten kinetics was obtained, giving K_m 0·40 mM and V_{max} 5 μmole $min^{-1}\ mg^{-1}$ with respect to PNBC. When the HPLC-assay derived enzymatic GSH + GST reactivity data cited in Section 3.1.1 were analysed as detailed in Section 2.3 for specific activity, a

TABLE 1
Representative Kinetic Data for PNBC

Assay[a,b,c]	*pH* (±0·2)	*T°C* (±3)	*Half-life* (*min*)	*Standard deviation*	*Standard deviation* (%)	*Number of assays*
GSH	7·0	22	480	120	25	50
GSH + GST	7·0	22	23	8	35	50
GSH + GST	7·0	38	17	8	50	100

[a] PNBC (0·1 mM).
[b] GSH (5 mM).
[c] GST (0·005 mg protein ml^{-1} assay solution).

value of 1 μmole min^{-1} mg^{-1} was obtained, in good agreement with 1·2 μmole min^{-1} mg^{-1} estimated from the derived K_m and V_{max} values using the spectrophotometric assay. Although V_{max}/K_m ratios are commonly used as a measure of enzyme catalytic efficiency, the restricted aqueous solubility of the majority of commercial and exploratory agrochemicals was judged to make routine measurement of these parameters impracticable.

3.1.3 Temperature and pH dependence

1-Chloro-3,4-dinitrobenzene and *N*,*N*-dipropyl-2-chloroacetamide were used as model compounds to determine the temperature and pH dependence of the non-enzymatic GSH assay. Representative data are given in Table 2. Excellent Arrhenius plots of second-order rate constant (M^{-1} s^{-1}) versus $1/T$ were obtained ($r^2 = 0{\cdot}998$), yielding activation energies (E_a) of 80·3 and 79·5 kJ mol^{-1}, respectively, for 1-chloro-3,4-dinitrobenzene and *N*,*N*-dipropyl-2-chloroacetamide. These values are similar to those published in a diverse range of hydrolysis studies.[16] In simple terms, a 10°C rise in temperature would be expected to give a *c.*3-fold increase in both hydrolysis and non-enzymatic reactivity with GSH. Note, however, that these two non-enzymatic reactions are not necessarily linked and can arise independently of each other.

Fits of second-order rate constant (M^{-1} s^{-1}) versus pH to the rate expression:

$$\log \text{rate (pH)} = \log \text{rate (maximum)} - \log[1 + 10^{(pKa - pH)}]$$

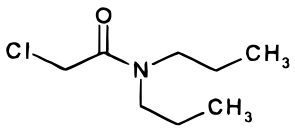
gave pK_a values of 9·9 and 9·8 respectively for 1-chloro-3,4-dinitrobenzene and *N*,*N*-dipropyl-2-chloroacetamide. These values represent the dissociation of GSH to the reactive thiolate anion GS^- in 50 mM phosphate buffer and 1 mM EDTA at 10°C (this temperature was selected to maximise the reliability of data measured at high pH). Less reliable data, obtained for 1-chloro-3,4-dinitrobenzene at 25°C yielded a pK_a of *c.*9·5. Such kinetic pK_a values for GSH will be a composite of its overlapping thiol and amine dissociation constants which will be dependent upon experimental conditions, particularly ionic strength and temperature. Although GSH is commonly cited as having pK_a *c.*9 for dissociation to GS^-, this work clearly indicates that it would be inappropriate to apply this value to kinetic data obtained under our experimental conditions.

Table 3 summarises results from a limited study of the temperature and pH dependence of PNBC reactivity in a single assay session. The 3-fold increase in non-enzymatic reactivity observed with GSH at 37°C, compared to 22°C, is in reasonable agreement with expectations from the model compound studies. The temperature dependence of the enzymatic GSH + GST reaction appeared to be significantly lower, with approximately a 2-fold increase in reactivity observed for a 15°C increase in temperature, consistent with the results summarised in Table 1.

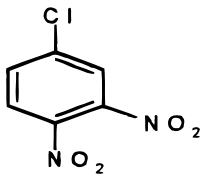
PNBC reactivity with GSH showed a similar pH dependence to the model compounds 1-chloro-3,4-dinitrobenzene and *N*,*N*-dipropyl-2-chloroacetamide. However, the enzymatic GSH + GST reactivity data are interesting in that they suggests that the catalytic efficiency of the enzyme, defined as the ratio of enzyme-catalysed to non-catalysed reaction rate, decreased from 4000 at pH 7·0 to zero at pH 10·5. This was not due to pH-induced inactivation of GST, since stock GST solutions (1 g $litre^{-1}$), prepared in buffers at pH 7·0, 9·0 and 10·5 then stored for >1 h at room temperature prior to use in enzymatic GSH + GST assays at pH 6·9 and 22°C, all gave half-lives of 17(±3) min with GSH (4·9

TABLE 2

Reactivity of Model Compounds with GSH: Temperature and pH Dependence



N,*N*-Dipropyl-2-chloroacetamide



1-Chloro-3,4-dinitrobenzene

Temperature T(°C)	*GSH assay*[a] $t_{1/2}$ *(min)*	*GSH assay* $(M^{-1}\ s^{-1})$	*pH* (±0·1)	*GSH assay*[b] $t_{1/2}$ *(min)*	*GSH assay* $(M^{-1}\ s^{-1})$
10	1777	0·0014	7·5	469	0·0053
25	374	0·0065	8·5	52	0·048
37	114	0·021	9·5	8·5	0·29
50	29	0·085	10·5	4·0	0·60
65	8	0·30	11·5	3·0	0·83

[a] GSH (4·72 mM) at pH 7·0 (±0·1).
[b] GSH (4·65 mM) at 10°C.

TABLE 3
Reactivity of PNBC with GSH and GSH + GST: Temperature and pH Dependence

pH (±0·1)	*T*(°C)	*GSH assay*[a] $t_{1/2}$ (*min*)	*GSH assay* ($M^{-1}\ s^{-1}$)	*GSH + GST assay*[a,b] $t_{1/2}$ (*min*)	*GSH + GST assay* [$M^{-1}\ s^{-1}\ (mg\ ml^{-1})^{-1}$]
6·9	22	434	0·0053	30	20
8·9	22	11	0·21	7	—
10·5	22	4	0·54	4	—
7·0	22	433	0·0053	29	25
7·0	37	144	0·016	15	47

[a] GSH (*c*.4·7 mM).
[b] GST (*c*.0·004 mg protein ml^{-1} assay solution).

mM) and GST (0·0043 mg ml^{-1}). It is established that mammalian GSTs facilitate the dissociation of GSH at physiological pH values, the relevant pK_a for GSH on binding to rat liver GSTs being decreased from *c*.9 to *c*.6 to 7, dependent on isoform.[17,18] The method of isolation and purification used for the commercial source of rat liver GST used in our work gives a multi-isoform preparation,[19] our GSH and GSH + GST results for PNBC being consistent with the pK_a range cited for GSH bound to GSTs.

3.1.4 Co-solvent dependence

The model compounds and commercial agrochemicals studied in this work were sufficiently water-soluble (>1 mg $litre^{-1}$) to allow measurements of hydrolysis, non-enzymatic GSH and enzymatic GSH + GST reactivity using aqueous buffers. However, commercial and exploratory agrochemicals can have significantly lower water-solubilities and/or weak chromophores leading to poor detection by HPLC. Consequently, the influence of organic co-solvents on the reactivity of the standard PNBC in the enzyme-catalysed GSH + GST assay was investigated. Aqueous pH 7·0 buffer + organic medium (80 + 20 by volume) led to a *c*.50% decrease in reactivity using methanol, ethanol, propan-1-ol, propan-2-ol, acetone, acetonitrile and dimethylformamide, compared to 100% aqueous buffer assays at 20–25°C. On decreasing the organic co-solvent content to 10% by volume, ethanol was identified as the preferred co-solvent, as it retained *c*.90% of the reactivity of the 100% aqueous control, the other solvents giving 20–30% loss of reactivity. The interpretation of kinetic data from pH-dependent hydrolysis and GSH reactions in mixed solvents is known to be problematic,[11,16] consequently no studies relating to the use of co-solvents in these non-enzymatic assays were made.

3.2 Hydrolysis, non-enzymatic GSH and enzymatic GSH + GST reactivity of agrochemicals

Measurements of hydrolysis, non-enzymatic GSH and enzymatic GSH + GST reactivity were made for a diverse range of agrochemicals, chosen mainly on the basis of literature precedent for the involvement of GSH and GSTs in their metabolism.[3] Table 4 summarises data and Fig. 1 gives the structures of those agrochemicals for which reactivity with GSH was determined in the presence and absence of GST. Also included are reference data for the assay standard 4-nitrobenzyl chloride (PNBC) and the standard 1-chloro-2,4-dinitrobenzene (CDNB) commonly used by other workers.[12] The enzymatic GSH + GST assays were normally run at pH 7·0 using GSH (5 mM), GST (0·09 mg ml^{-1} assay solution) and compound (2–100 μM, depending on solubility). Corrections of enzymatic GSH + GST data for non-enzymatic GSH reactivity or hydrolysis were usually <10% of the enzyme-catalysed rate. Further optimisation of GSH + GST relative to GSH reactivity and/or hydrolysis was normally obtained by varying the GST concentration (0·005 to 0·94 mg ml^{-1} assay solution).

For triclopyr, halosulfuron-methyl and azoxystrobin, rates of hydrolysis and GSH reactivity could not be differentiated reliably under the conditions shown and therefore all non-enzymatic reactivity was assigned to hydrolysis. The enzymatic GSH + GST reactivity data for triclopyr and azoxystrobin did not need correction for hydrolysis, but under the normal assay conditions used for halosulfuron-methyl, the first-order rate constant for the enzyme-catalysed reaction required a correction of *c*.60% for hydrolysis prior to calculation of second-order related rate constant and specific activity. For most compounds in Table 4, a direct measurement of non-enzymatic reactivity with GSH was obtained through variation of temperature (20–40°C) and/or pH (7–11). For more detailed studies of structure–activity and structure–reactivity relationships of exploratory agrochemicals, the temperature and pH relationships derived in Section 3.1.3 can be applied to such measurements to calculate non-enzymatic GSH second-order rate constants under the experimental conditions used for the enzymatic GSH + GST assays. These calculations then allow comparison of uncatalysed GSH and catalysed GSH + GST reactivity data for a given temperature and pH, and an estimation of catalytic efficiency for GSTs.

TABLE 4
GSH and GSH + GST Reactivity Data for Agrochemicals and Standards

Compound	*pH* (±0·2)	*T*(°C) (±3)	*GSH assay* [a,b] $t_{1/2}$ (*min*)	*GSH assay* [b] (M^{-1} s^{-1})	*GSH + GST assay* [a,c] $t_{1/2}$ (*min*)	*GSH + GST assay* [c] (M^{-1} s^{-1} (*mg ml*$^{-1}$)$^{-1}$)	*GSH + GST* [a,c] *2nd-order rate ratio to PNBC*	*GSH + GST* [a,c] *assay* (*nmole*$^{-1}$ *min*$^{-1}$ *mg*$^{-1}$)
CDNB	7	22	200 (±70)	0·012	8	396	20	11 600
PNBC[d]	7	22	480 (±120)	0·005	23 (±8)	25	1·0	1000
Acetochlor	7	22	—	—	68	1·7	0·042	33
	9	37	15	0·17	—	—	—	—
Alachlor	7	22	960 (±110)	0·0024	30 (±13)	3·7	0·10 (±0·03)	72 (±19)
	9	37	8	0·31	—	—	—	—
Butachlor	7	20	—	—	345 (±149)	0·35	0·012 (±0·009)	3·3 (±1·6)
	9	20	43 (±6)	0·057	—	—	—	—
Metolachlor	7	20	—	—	382	0·30	0·0076	5·7
	9	37	70 (±9)	0·035	—	—	—	—
Propachlor	7	20	—	—	61 (±6)	0·83	0·038 (±0·004)	21 (±3)
	9	25	5	0·54	—	—	—	—
Pretilachlor	7	40	251	0·0098	89	0·30	0·0060	4·3
Dichlormid	7	37	—	—	256 (±69)	0·16	0·0032 (±0·0003)	2·8 (±1·4)
	9	37	634	0·0039	—	—	—	—
Flurochloridone	7	37	—	—	581 (±181)	0·081	0·0035 (±0·0013)	1·2
	10	37	798	0·0029	—	—	—	—
Fluazifop-butyl	7	37	—	—	62 (±22)	0·42	0·0071 (±0·001)	0·26 (±0·03)
Fluorodifen	7	25	2300 (±800)	0·0010	23	1·76	0·028	2·4
	10·5	26	215	0·011	—	—	—	—
Fomesafen	7	37	—	—	2220	0·016	0·000 56	0·16
Azoxystrobin	7	33	—	—	653	0·0052	0·000 14	0·035
	9	37	6000 (±2000)	0	—	—	—	—
Atrazine	7	23	—	—	419	0·0084	0·000 14	0·21
	10·5	37	459	0·0050	—	—	—	—
Halosulfuron-methyl	7	36	5300 (±900)	0	3320	0·0049	0·000 08	0·029
	9	36	250 (±50)	0	—	—	—	—
Triclopyr	7	37	2100 (±600)	0	57	0·35	0·0058	0·22
Tridiphane	7	25	880 (±290)	0·0026	23 (±5)	9·0	0·15 (±0·1)	15
	9	26	76	0·032	—	—	—	—
Chlorothalonil	7	36	3	0·78	0·5	86	2·5	74
Fluazinam	7	40	22	0·11	<2	*c.* 2500	*c.* 50	*c.* 50 000
Diazinon	7	23	—	—	388	0·089	—	1·6

[a] Mean (±maximum deviation) from two to four replicates.
[b] — (dash) indicates no reactivity observed.
[c] — (dash) indicates no data.
[d] See sections 3.1.1 and 3.1.2.

Enzymatic GSH + GST reactivities expressed in second-order related rate constant terms, ratioed to the standard PNBC to provide a value independent of assay conditions, spanned five orders of magnitude. For the agrochemicals, reactivity varied over 1000-fold, with significant differences in reactivity observed within the chloroacetanilide and diphenyl ether type, chemical classes.

The chloroacetanilide herbicides, which on reaction to form GS-conjugate displace chloride from the α-chloro-carbon electrophilic site, had a *c.*15-fold spread in reactivity, with alachlor being the most and pretilachlor, metolachlor and butachlor the least reactive. The dichloroacetamide herbicide safener dichlormid and the herbicide flurochloridone (which contains the 3-chloro-4-chloromethyl-2-pyrrolidinone structural moiety) are related to the chloroacetanilides with respect to electrophilic site and were *c.*2-fold less reactive than metolachlor. The rate of GSH conjugation of alachlor by rat liver cytosolic enzymes is reported to be 20 times faster than that of metolachlor,[20] in reasonable agreement with the 13-fold difference shown in Table 4.

The reactivities of the diphenyl ether type herbicides, which cleave at the electrophilic carbon ether linkage to give phenols and GS-aryl or GS-heterocycle conjugates, overlapped the chloroacetanilide range. In terms of enzymatic GSH + GST reactivity, fluorodifen was similar to acetochlor and fluazifop-butyl similar to metolachlor. Fomesafen was 50-fold less reactive than fluorodifen and 4-fold more reactive than the fungicide azoxystrobin, which has two phenylheterocyclic ether moieties. Triclopyr, atrazine and halosulfuron-methyl react at heterocycle carbon-chloride electrophilic sites to displace chloride and form GS-heterocycle conjugates. Triclopyr was slightly less reactive than metolachlor, while atrazine and halosulfuron-methyl had low levels of reactivity, similar to that of azoxystrobin. Tridiphane, in which the epoxide is opened to give the GS-conjugate, was the most reactive herbicide, with slightly higher reactivity than alachlor. The fungicides chloro-

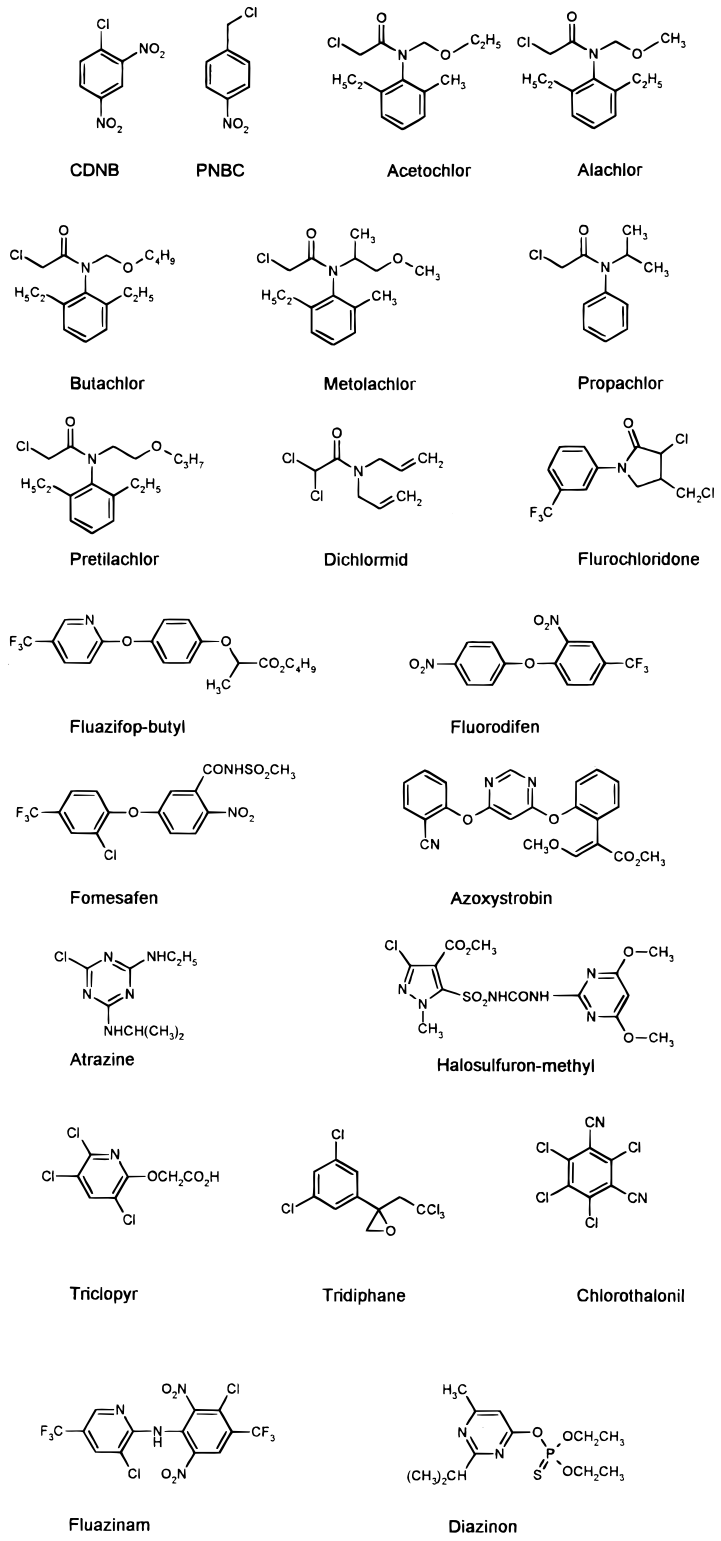


Fig. 1. Structures of agrochemicals and model compounds.

thalanil and fluazinam, which react at phenyl carbon-chloride electrophilic sites to displace chloride and form GS-phenyl conjugates, had high levels of reactivity with both GSH alone and with GSH + GST. Fluazinam's enzyme-catalysed reactivity with GSH + GST was 2·5 times faster than the commonly used standard 1-chloro-2,4-dinitrobenzene, consistent with the presence of a closely related structural moiety in fluazinam.[21] Diazinon, an organophosphorus insecticide which cleaves on reaction to give diethyl phosphorothioic acid and a GS-pyrimidine conjugate,[22] had a similar level of reactivity to that of the herbicide flurochloridone.

4 DISCUSSION

The HPLC-based non-enzymatic GSH and enzymatic GSH + GST assay system was developed primarily to provide metabolism-related reactivity parameters to aid the exploration of structure–activity and structure–reactivity relationships in support of agrochemical research programmes. The variations in experimental conditions used and the range of reactivity data obtained in this work attest to the versatility and sensitivity of the HPLC-based assay system.

With respect to structure–activity analysis, parameters based on second-order related rate constants from non-enzymatic GSH and enzymatic GSH + GST assays have considerable potential to provide insights into the role of both GSH- and GST-mediated metabolism with respect to biological activity. Measures of reactivity for an individual exploratory lead, though useful, are considered to be of limited value given the broad range of reactivities measured for commercial agrochemicals. The preferred approach has been to generate a database of comparative reactivity values for exploratory leads, analogues and relevant commercial products. Correlations are then sought with herbicidal, fungicidal and insecticidal activity within and across biological screens to investigate issues such as translation of in-vitro to in-vivo activity and in-vivo activity spectrum and selectivity. The generation of these chemical or biological target-specific databases gives considerable scope for developing predictive structure–reactivity relationships within and across chemical classes. In combination, such databases currently provide Zeneca Agrochemicals with reactivity parameters for more than 500 model, exploratory and commercial agrochemicals.

Although a commercial multi-isoform rat liver GST preparation is routinely used to assess intrinsic reactivity, the enzymatic GSH + GST assay has the potential to utilise GSTs of diverse source and purity. Application of the assay system to characterise the substrate specificity of glutathione transferase isoforms I and II from maize with respect to 1-chloro-2,4-dinitrobenzene, alachlor, fluorodifen and atrazine has been reported.[23] Soluble protein extracts from *c.*20 plant and insect species have also been used as the GST source for enzymatic GSH + GST assays using a range of compounds representing different chemical classes. This work will be reported separately and compared with existing literature at that time. However, the overall conclusion drawn was that the rat liver GST preparation had a tendency to overestimate the potential of a compound to react with plant or insect GSTs, but, in broad terms, gave similar rankings of reactivity across different chemical classes. Consequently, we consider the commercially available rat liver GST preparation to be an appropriate choice for an intrinsic reactivity assay from which to derive metabolism-related parameters for use in structure–activity and structure–reactivity studies.

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